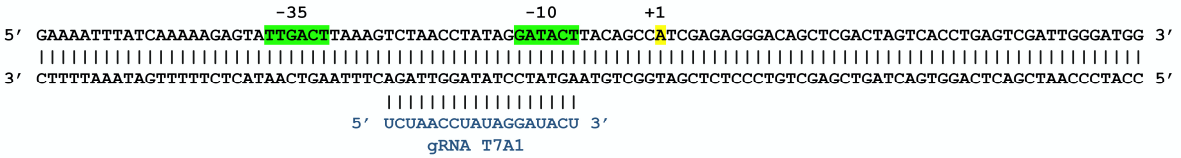
Recognition of double-stranded DNA by the *Rhodobacter sphaeroides* Argonaute protein

Lidia Lisitskaya, Ivan Petushkov, Daria Esyunina, Alexei Aravin, Andrey Kulbachinskiy

Supplementary Material

T7A1-short (tDNA+ntDNA, 102 bp):



T7A1-short-bubble (tDNA+ntDNA, 102 bp):

T7A1-long (PCR-product, 249 bp):

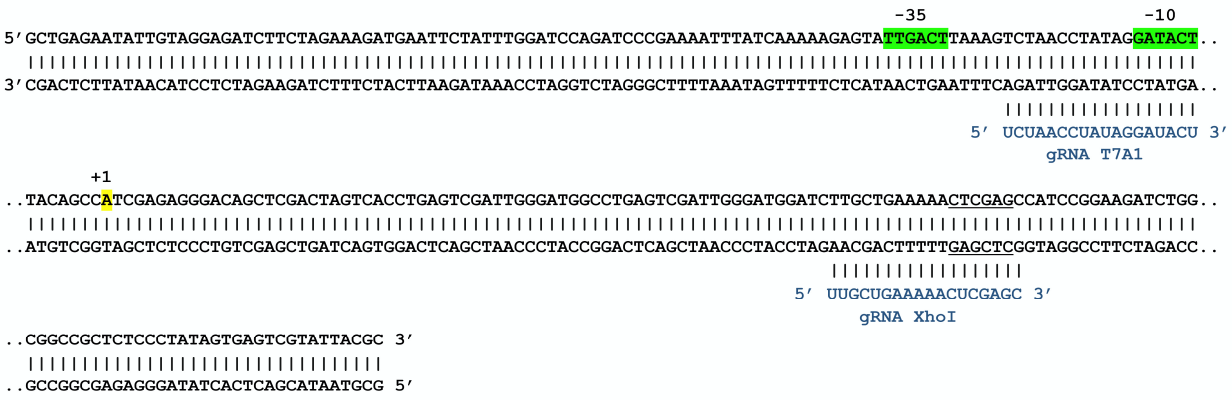


Figure S1. Sequences of DNA targets and RNA guides used for analysis of DNA targeting by RsAgo. gRNAs are shown in blue, the noncomplementary region in the T7A1-short-bubble template is orange, the XhoI site in T7A1-long is underlined. The -10 and -35 promoter elements are shown in green, and the transcription start site is yellow.

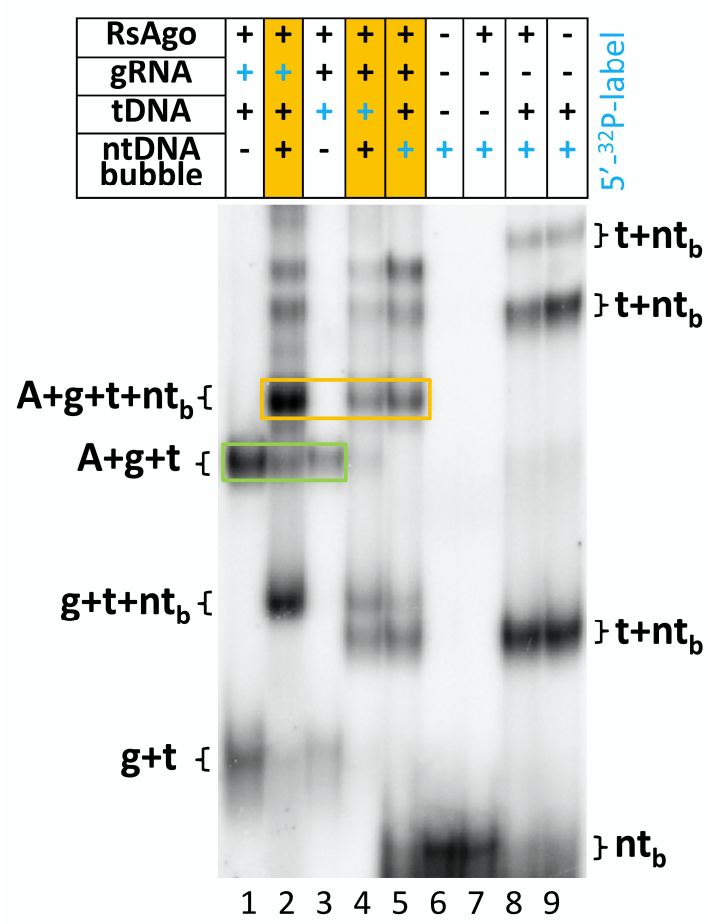


Figure S2. Reconstitution of RsAgo complexes with target dsDNA containing a partially melted region (T7A1-short-bubble). The tDNA strand (50 nM) was pre-annealed with the ntDNA strand (75 nM), followed by the addition of gRNA-loaded RsAgo (400 nM gRNA, 300 nM RsAgo) at 30 °C. The samples were analyzed by non-denaturing 7% PAGE followed by phosphorimaging. The 5'-labeled component in each reaction is shown in blue. Positions of free oligonucleotides and their complexes with RsAgo are indicated (ntDNA strand with a noncomplementary region corresponding to the site of gRNA binding is indicated as "nt_b", for "nontemplate bubble", see Fig. S1). Position of the quaternary complex of RsAgo with gRNA and dsDNA is shown in orange; position of the ternary complex of RsAgo with gRNA and tDNA is shown in green.

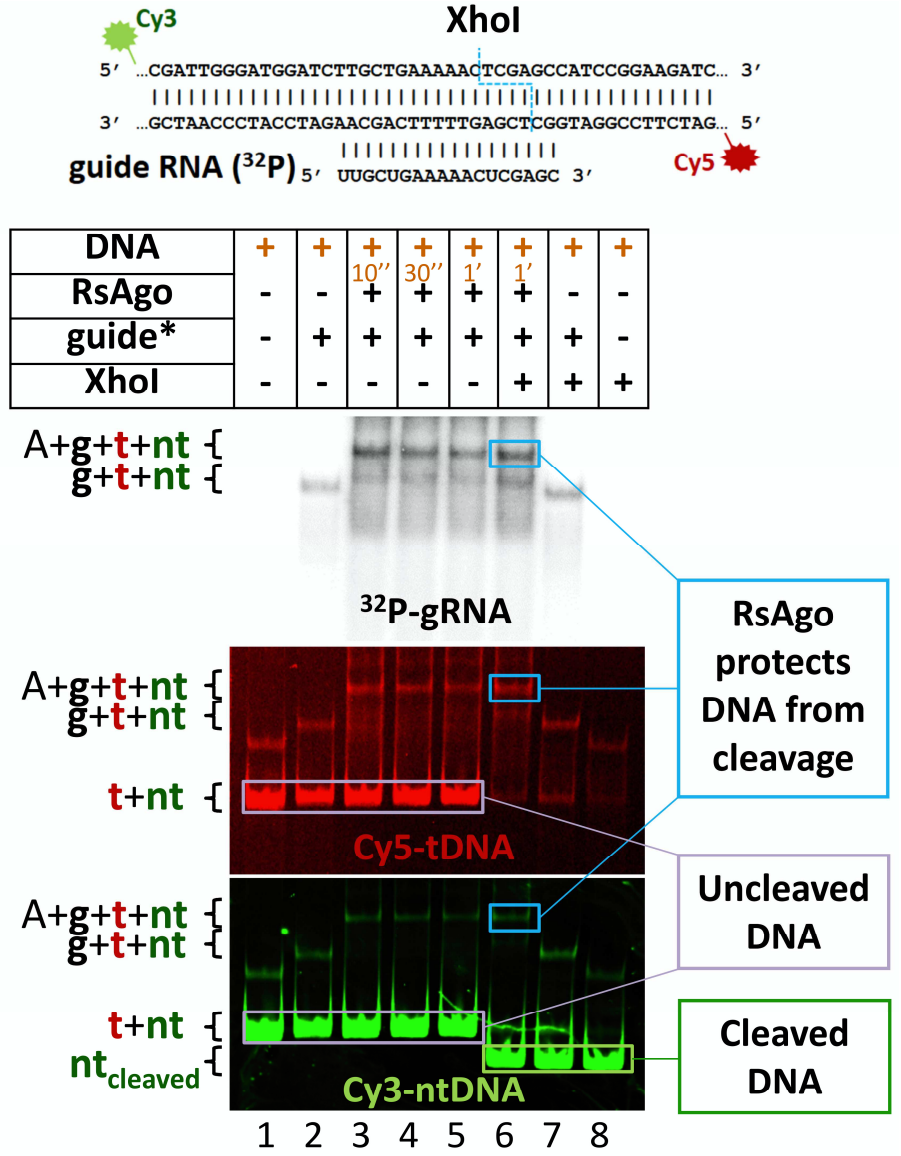


Figure S3. RsAgo protects DNA from endonuclease cleavage. The sequence of the target DNA (T7A1-long) is shown in Fig. S1; gRNA, tDNA and ntDNA were 5'-labeled with ^{32}P-, Cy5 and Cy3, respectively. DNA was premelted at 95°C and transferred to 40°C for 10", 30" or 1', followed by the addition of guide-loaded RsAgo (lanes 3-6) or gRNA alone (lane 2 and 7). The samples were incubated for 15 minutes at 40°C for complex formation, treated with XhoI when indicated (lanes 6-8) and analyzed by non-denaturing PAGE. The ^{32}P-, Cy5 and Cy3 labels were visualized with a Typhoon 9500 scanner. Positions of uncleaved and cleaved target dsDNA, as well as the position of gRNA-RsAgo-dsDNA complexes protected from the XhoI cleavage (and containing all three labels, ^{32}P-, Cy5 and Cy3) are indicated. Note that the shorter cleavage product of the T7A1-long DNA containing the Cy5 label in the target strand is not visible because it has run out of the gel.

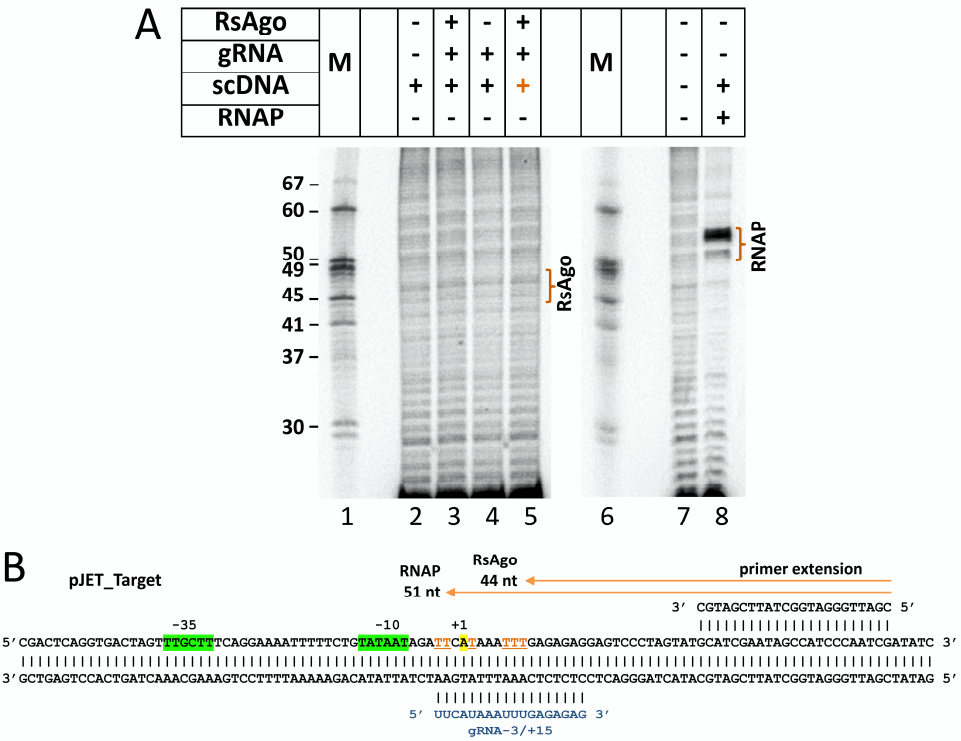


Figure S4. Guide-loaded RsAgo does not form specific complexes with supercoiled plasmid DNA *in vitro*. (A) $KMnO_4$ footprinting of the target DNA region in reactions containing RsAgo (lanes 2-5) or *E. coli* RNA polymerase (lanes 7, 8). Lanes 1 and 6 contain DNA size markers. (B) Scheme of the target DNA region and positions of gRNA and the primer used for the primer extension reaction.

To detect the interactions of RsAgo with a supercoiled plasmid, we used $KMnO_4$ footprinting followed by primer extension for identification of the melted region. A DNA fragment containing the N25 promoter of phage T5 and a target site recognized by "gRNA -3/-15" was cloned into the pJET1.2 vector and the plasmid was isolated by GeneJET Plasmid Miniprep Kit (Thermo Scientific). RsAgo (500 nM) was mixed with 5'-phosphorylated gRNA (200 nM) and incubated at 30 °C for 10 min. Supercoiled plasmid (20 nM) was directly added to the binary complex or was preheated at 98 °C for 2 min (indicated with red), rapidly transferred to 0 °C for 10 sec and added to the binary complex, and the samples were incubated at 30 °C for 15 min. The samples were treated with $KMnO_4$ and then with chloroform as described in Materials and Methods. DNA was ethanol precipitated, dissolved in water (MilliQ) and treated with XhoI, to cut plasmid DNA upstream of the N25 promoter and prevent the formation of long primer extension products. PCR was performed with 1 unit of Taq polymerase with 1 µM of 5'-^{32}P-labeled primer in Taq-buffer with $(NH_4)_2SO_4$ (Thermo Scientific) under the following conditions: 95 °C 5 min, 95 °C 30 sec, 63 °C 30 sec, 72°C 15 sec, 20 cycles. The samples were treated with water-free chloroform, DNA from the water phase was ethanol precipitated and dissolved in 10 µl of water. The samples were mixed with an equal volume of formamide (98%), heated at 95 °C for 5 min and analyzed by 15% PAGE. To identify the position of thymine modifications, a marker with radioactively labeled DNA oligonucleotides was used (lane 1 in panel A). As a positive control, we tested RNA polymerase from *E. coli* for binding with the promoter region. To assemble the promoter complex, supercoiled plasmid (20 nM) was mixed with the core enzyme of RNA polymerase (300 nM) and the σ^{70} subunit (1 µM) in transcription buffer (Tris-HCl 40 mM, pH 7.9, NaCl 40 mM, $MgCl_2$ 10 mM) and incubated at 37 °C for 10 min. $KMnO_4$ footprinting of promoter complexes and primer extension was performed as described above. No modified thymine residues corresponding to the expected binding site of RsAgo could be detected, even if DNA was preheated before the addition of RsAgo. Thus, supercoiled plasmid DNA cannot be efficiently targeted by RsAgo under these conditions.